

# Silencing the expression of multiple G $\beta$ -subunits eliminates signaling mediated by all four families of G proteins

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The G $\beta\gamma$ -subunit complex derived from heterotrimeric G proteins can act to regulate the function of a variety of protein targets. We established lentiviral-based RNA interference in J774A.1 mouse macrophages to characterize the role of G $\beta$  in G protein-coupled receptor signaling. The expression of G $\beta$ 1 and G $\beta$ 2, the major subtypes present in J774A.1 cells, was eliminated by sequential treatment with small hairpin RNA expressing lentivirus. These  $\beta\gamma$  complex-deficient cells lost the ability to respond to G protein-mediated signals. Chemotaxis and the phosphorylation of Akt in response to C5a were both blocked. Similarly, C5a-mediated actin polymerization, C5a- and UTP-stimulated intracellular calcium mobilization, and the stimulation of cAMP formation by isoproterenol were all eliminated in the absence of the G $\beta$ -subunits. In addition, stabilization and membrane localization of several G $\alpha$ - and G $\gamma$ -subunit proteins was strongly effected. Furthermore, in DNA microarray analysis, regulation of gene expression stimulated by prostaglandin E2 and UTP was not observed in cells lacking G $\beta$ -subunits. In contrast, phagocytotic activity, serum-dependent cell growth and the patterns of gene expression induced by stimulating the Toll receptors with LPS were similar in wild-type cells and small hairpin RNA-containing cells. Thus, ablation of the G $\beta$ -subunits destabilized G $\alpha$ - and G $\gamma$ -subunits and effectively eliminated G protein-mediated signaling responses. Unrelated ligand regulated pathways remained intact. These cells provide a system that can be used to study signaling in the absence of most G protein-mediated functions.

cell signaling | RNA interference

Upon interaction of G protein-coupled receptors (GPCRs) with their cognate ligands, heterotrimeric G proteins are activated and dissociate into a GTP-bound  $\alpha$ -subunit and a  $\beta\gamma$  dimer, and both of these can regulate specific downstream effector molecules (1, 2). The effector molecules regulated by G $\beta\gamma$  in mammals include certain isoforms of adenylyl cyclase, phospholipase C- $\beta$  (PLC- $\beta$ ), phosphatidylinositol 3-kinase, p21-activated kinase, and GPCR kinases (3–7). In addition, G $\beta\gamma$  has been shown to modulate the activity of various ion channels (8, 9). The G $\beta\gamma$ -subunits are always found in the dimeric form, and the interaction of the G $\beta$  protein with the G $\gamma$  protein appears to be required for their mutual stability (10). The G $\gamma$ -subunit contains a CAAX motif at its carboxyl terminus that directs lipid modification and is required for membrane localization of the G $\beta\gamma$  dimer (11).

The function of the G $\beta$  molecule has mainly been studied in cell-free systems or on the cellular level. These studies have helped to define the nature of G $\beta$  interactions with other molecules (12). A genetic approach to the study of the question of G $\beta$  function was conducted in *Dictyostelium*, which contains a single G $\beta$ -subunit (13). The G $\beta$ -null mutants were severely defective in development, chemotaxis, and phagocytosis. Genetic analysis has also been useful in establishing some of the functions of the G $\beta$ 1-subunit in *Caenorhabditis elegans*. Thus, for example, Zwaal *et al.* (14) showed that expression of G $\beta$ 1 was important for orientation of the early cell division axis in the first

larval stage. However, it is difficult to study the roles of the G $\beta$  family members in mammalian systems by using genetic techniques because mammalian cells express multiple G $\beta$  subtypes that can have similar or overlapping functions (15). However, gene silencing techniques employing double-stranded small interfering RNA (siRNA) can be used to observe the effects of specific target protein depletion on cellular function (16, 17). Furthermore, lentiviral vectors containing a selection marker, e.g., an antibiotic resistance gene, have been shown to act as efficient means for delivering siRNA (15, 18). They insert into the cellular genome and can be used to generate cell lines that express a specific siRNA as a hairpin structure (shRNA). In the present study, we demonstrate the effects of eliminating individual G $\beta$  isoforms, or all G $\beta$  isoforms, by lentivirus-delivered shRNAs. We conclude that the G $\beta$ -subunits modulate all of the GPCR-related cellular responses that we measured. Interestingly, the elimination of a functional G protein-signaling system leaves other signal transduction pathways intact.

## Materials and Methods

**Virus Generation.** Most lentiviral vectors were constructed and used for virus generation as described (15, 19). To make pL-UGIH, puromycin-resistance gene sequences were replaced with hygromycin-resistance gene sequences in pL-UGIP.

Adenovirus-expressing recombinant G $\alpha$ q proteins were generated through homologous recombination between a linearized transfer vector pAD-Track CMV and the adenoviral backbone vector pAD-easy1 as described (20). The adenoviral vector contained either the wild-type G $\alpha$ q cDNA (GqW) or the myristylation mutant G $\alpha$ q cDNA (GqAG) with a Glu-Glu (EE) epitope in the internal recognition site (171–176 residues) (21). Expression of recombinant proteins in the virus-infected cells was verified by immunoblotting with anti-EE antibody (Novus Biologicals).

**Western Blotting.** Western blotting was performed with the following antibodies. Anti-phospho Akt antibody (raised against pSer473-Akt) (Cell Signaling Technology), anti-tubulin antibody (Oncogene), antibodies for Gai2 and G $\alpha$ q (kind gift from Susanne Mumby, University of Texas Southwestern Medical Center, Dallas), anti-G $\gamma$ 12 antibody (kind gift from Tomiko Asano, Aichi Human Service Center, Aichi, Japan) (22), anti-C5a receptor (Pharmingen), and other anti-G protein antibodies (Santa Cruz Biotechnology) were used to detect their corresponding proteins. Peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies (Amersham Pharmacia) were used, and then the proteins were detected by using an enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia).

Abbreviations: GPCR, G protein-coupled receptor; PLC, phospholipase C; siRNA, small interfering RNA; shRNA, small hairpin RNA; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup>.

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**Measurement of Intracellular  $\text{Ca}^{2+}$  Mobilization and cAMP Assay.** Cells ( $2 \times 10^4$  per well) were grown in 96-well black wall/clear bottom plates (Corning) overnight. After incubation with  $1 \mu\text{M}$  fura-2/AM in Hanks' balanced salt solution (HBSS) for 30 min, cells were washed with HBSS twice. The ratio of fluorescence emission at 510 nm after excitation at 340 nm and 380 nm, caused by intracellular  $\text{Ca}^{2+}$  mobilization, was determined by using the Flexstation, a fluorometric imaging plate reader (Molecular Devices). cAMP generation was measured by using a HitHunter cAMP assay kit as described by the manufacturer (Applied Biosystems).

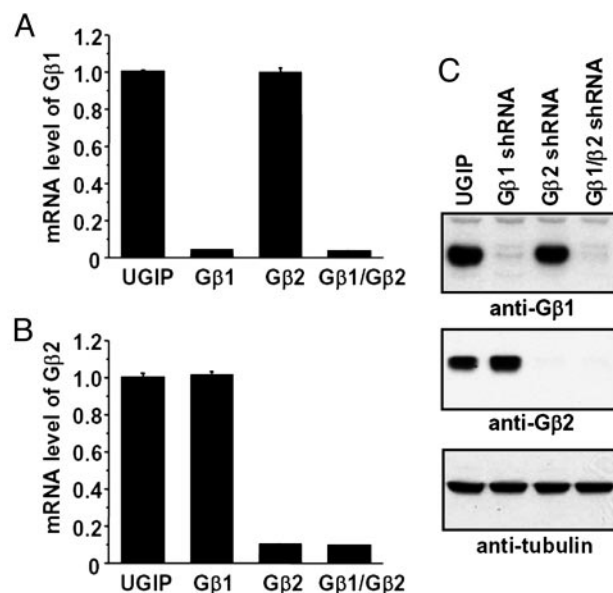
**Chemotaxis and Actin Polymerization.** The *in vitro* cell migration assay was performed as described (15). J774A.1 cells were plated on poly(L-lysine)-coated cover slips and incubated overnight in culture medium. After 6-h serum starvation, cells were stimulated with C5a for 2 min. They were fixed by using 4% paraformaldehyde in PBS for 30 min. Cells were permeabilized with 0.1% Triton X-100, stained with 0.5 unit of Texas red-conjugated phalloidin (Molecular Probes), and analyzed by confocal microscopy (Leica).

**Cellular Fractionation.** Cells were harvested with hypotonic buffer (20 mM Hepes, pH 7.5/3 mM  $\text{MgCl}_2$ /10 mM KCl) containing protease inhibitors, homogenized by passing through a 26-gauge needle 10 times. Lysates were centrifuged at  $1,000 \times g$  for 10 min to remove unbroken cells and nuclei. The cleared lysates were subject to ultracentrifugation at  $100,000 \times g$  for 1 h. The supernatants were kept as the cytosolic fractions. The precipitates which were kept as the membrane fraction were washed with same buffer twice and sonicated in a half volume of RIPA buffer containing protease inhibitors. Thirty micrograms of each fraction was then loaded on SDS/PAGE, followed by Western blotting with appropriate antibodies.

**DNA Microarray.** After growth in media containing 0.5% FBS for 18 h, cells were stimulated with agonist for 1 or 4 h, and then harvested with Trizol (Invitrogen). Total RNAs from the cells were used for microarray analysis. The 16,000 mouse oligonucleotide arrays were inkjet-printed by Agilent Technologies. These arrays include 13,536 70mers (Operon Technologies Inc.) and 2,304 65mers (Sigma-Genosys). The aminoallyl method was used for the preparation of fluorescently labeled target samples. All procedures and data analysis methods are described on the web site of the Alliance for Cellular Signaling ([www.signaling-gateway.org/data/ProtocolLinks.html](http://www.signaling-gateway.org/data/ProtocolLinks.html)).

## Results

**Establishment of Cells Lacking G $\beta$ 1 and G $\beta$ 2 Proteins.** Transfection of J774A.1 cells with plasmid-based expression vectors is very inefficient (15). However, pseudotyped lentivirus, which efficiently infects these cells and can be integrated into chromosomes, is a good carrier for delivery and sustained expression of genes (18). We constructed lentiviral vectors with cassettes that allow the expression of GFP and antibiotic selection markers to identify and enrich the fraction of transduced cells. We have shown in previous studies that G $\beta$ 1 and G $\beta$ 2 are the major abundant G $\beta$  proteins in J774A.1 cells; the other isoforms were not detected in immunoblot analysis (15). A very low level of G $\beta$ 4 mRNA was found in RT-PCR experiments, but no G $\beta$ 4 protein was detected by anti-G $\beta$ 4 antibody. To establish cell lines expressing two siRNA genes, two cycles of infection and selection were performed. In the first cycle, cells were infected with a virus containing the G $\beta$ 1 shRNA that we previously demonstrated to be effective (15) and an expression cassette for puromycin resistance. The cells were then selected by growth in puromycin. In the second cycle, puromycin-resistant cells were infected with a virus containing the G $\beta$ 2 shRNA expression construct and a gene expressing hygromycin resistance. They

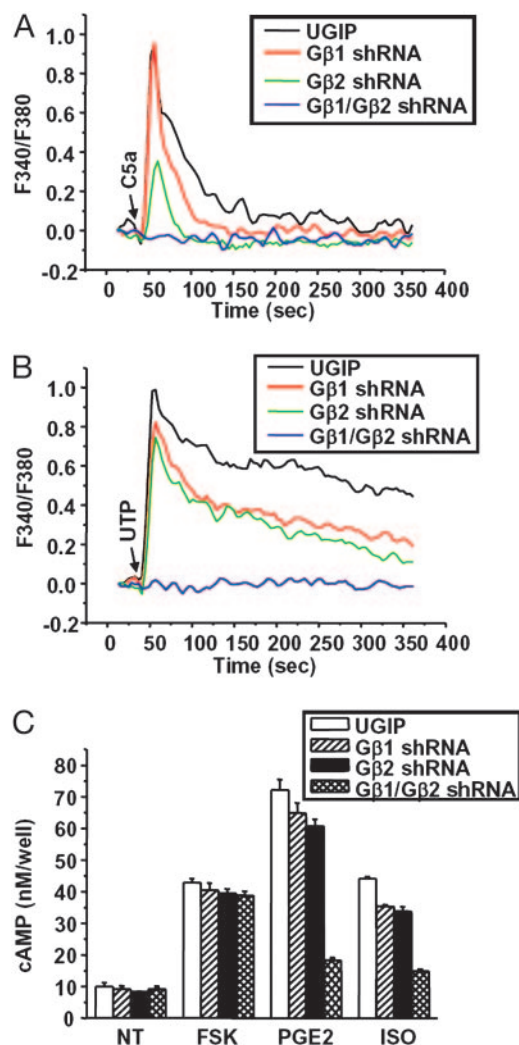


**Fig. 1.** shRNA-mediated inhibition of endogenous G $\beta$  expression in J774A.1 mouse macrophages. (A and B) After lentivirus infection and puromycin selection for G $\beta$ 1 and G $\beta$ 2 or two cycles of infection and selection with puromycin and hygromycin for G $\beta$ 1/G $\beta$ 2, mRNA levels of G $\beta$ -subunits were analyzed by TaqMan RT-PCR. mRNA levels were normalized to GAPDH mRNA. (C) Western blotting using subtype-specific antibodies. Protein expression was normalized with tubulin levels in total cell extracts.

were then grown and selected with hygromycin. The specific gene silencing activity of shRNA in the J774A.1 cells was confirmed by quantitative RT-PCR with specific primers and immunoblotting for endogenous proteins with isotype-specific antibodies. The mRNA level of each gene, normalized with GAPDH mRNA, was decreased by  $>90\%$  in the doubly infected cells (Fig. 1A and B). Consistent with RT-PCR data, significant decreases and inability to detect levels of G $\beta$ 1 and G $\beta$ 2 proteins were observed in Western blots (Fig. 1C). Nonetheless, when we transferred the cells to new plates and cultured at high density, wild-type and G $\beta$  knockdown cells showed similar growth rates. At low plating densities the G $\beta$  depleted cells showed slightly lower viability (data not shown).

**Intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) Transients and cAMP Generation Were Abolished in Cells Depleted of G $\beta$ -Subunits.** Increase of cytoplasmic  $\text{Ca}^{2+}$  concentration by GPCRs is known to be triggered by at least two different signaling pathways initiated by components of heterotrimeric G proteins, the G $\alpha_q$ - and G $\beta\gamma$ -subunits. Upon treatment with chemokines or with C5a, G $\beta\gamma$ -subunits released from the GTP-bound forms of G $\alpha_i$  increase cytoplasmic  $\text{Ca}^{2+}$  concentration by activating PLC- $\beta$ 2 or PLC- $\beta$ 3 (23). C5a-induced  $[\text{Ca}^{2+}]_i$  increases in a pertussis toxin-sensitive manner (Fig. 2A and previous reports). G $\beta$ 2 shRNA-containing cells showed a weak response (peaking at approximately half the level of control cells and of G $\beta$ 1 shRNA-containing cells), suggesting that G $\beta$ 2 may be the major transducer of C5a receptor signaling. When we treated G $\beta$ 1 and G $\beta$ 2 shRNA-containing cells with C5a, no  $[\text{Ca}^{2+}]_i$  release response was observed (Fig. 2A).

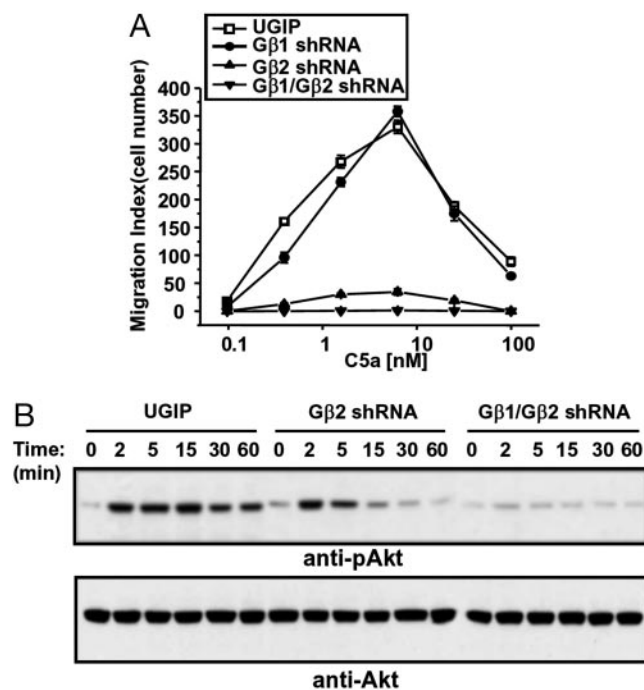
The P2Y receptors, a family of nucleotide receptors, respond to ligand binding by increasing  $[\text{Ca}^{2+}]_i$  release. They are thought to activate G $\alpha_q$  which in turn directly stimulates PLC- $\beta$  activation (24). Compared with the C5a-dependent  $[\text{Ca}^{2+}]_i$  response, UTP, a ligand interacting with P2Y receptors stimulated more potent and sustained responses in J774A.1 cells. The  $[\text{Ca}^{2+}]_i$  responses were slightly down-regulated in the cells containing single shRNA directed against G $\beta$ 1 alone or against G $\beta$ 2 alone. However, cells



**Fig. 2.**  $\text{Ca}^{2+}$  mobilization and cAMP production. Arrows indicate the time points that stimulants were added. (A and B) The effect of  $\text{G}\beta$  shRNA on  $[\text{Ca}^{2+}]_i$  changes stimulated by GPCR ligands. Cells were stimulated with 20 nM C5a (A) or 10  $\mu\text{M}$  UTP (B), and  $[\text{Ca}^{2+}]_i$  was monitored fluorometrically by using Fura-2/AM as described in *Materials and Methods*. Black, pL-UGIP (control); red, Gβ1 shRNA; green, Gβ2 shRNA; blue, Gβ1/Gβ2 shRNA. (C) cAMP production in the cells containing shRNA. Cells were stimulated with 10  $\mu\text{M}$  forskolin, 10  $\mu\text{M}$  PGE<sub>2</sub>, or 1  $\mu\text{M}$  isoproterenol for 10 min, and cAMP production was measured as described in *Materials and Methods*. Relative light units from experiment were converted to cAMP concentration by using standard curve per the manufacturer's description.

depleted of both Gβ1 and Gβ2 by the presence of both shRNAs showed no detectable  $[\text{Ca}^{2+}]_i$  response to UTP exposure (Fig. 2B). There is a large body of evidence indicating that different  $\beta\gamma$  combinations can substitute for each other in activating PLC (25). The loss of the  $[\text{Ca}^{2+}]_i$  response in doubly depleted cells suggests that the level of  $\beta\gamma$  has been reduced by the absence of both Gβ1 and Gβ2, so that no effective response normally stimulated by a GPCR through a heterotrimeric complex of  $\alpha$  and  $\beta\gamma$  can be mounted. To exclude the possibility that the lack of ligand-stimulated  $[\text{Ca}^{2+}]_i$  release in the absence of Gβ was due to shortage of intracellular  $\text{Ca}^{2+}$ , we evaluated the integrity of the  $\text{Ca}^{2+}$  stores. Thapsigargin induced similar  $[\text{Ca}^{2+}]_i$  increases in all cell types (data not shown), indicating that  $\text{Ca}^{2+}$  stores were not influenced by shRNA or by absence of Gβ-subunits.

$\text{G}\alpha\text{s}$ -mediated signaling modulates immune cell responses by regulating downstream signaling events and chemokine produc-



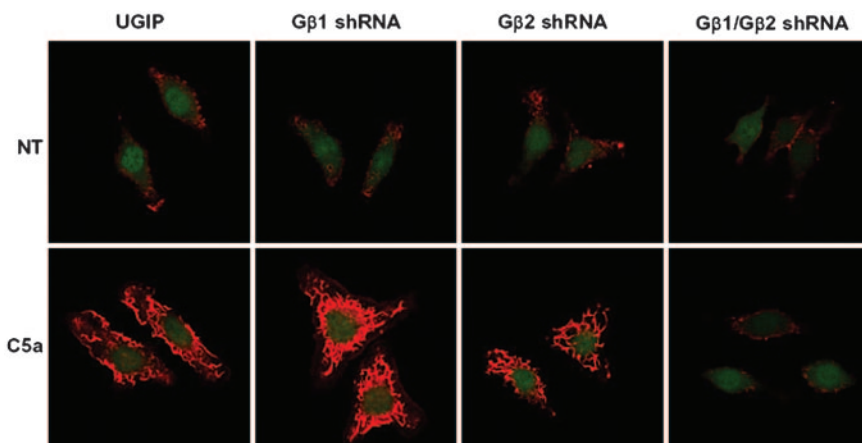
**Fig. 3.** Effects of  $\text{G}\beta$  silencing on cell responses to chemokines. (A) Chemotaxis of J774A.1 cells toward C5a. Chemotaxis was analyzed by using transwell migration, as described in *Materials and Methods*. The data are expressed as the mean  $\pm$  SE of three independent experiments. Open square, UGIP (control); filled circle, Gβ1 shRNA; filled triangle, Gβ2 shRNA; inverted filled triangle, Gβ1/Gβ2 shRNA. (B) C5a-stimulated phosphorylation of Akt. After incubation with media containing 1% FBS for 12 h, cells were stimulated with C5a for the indicated times. Cell lysates were subjected to immunoblotting with anti-phospho Akt antibody. Immunoblots were stripped and reprobed with anti-Akt antibody.

tion (26, 27). According to gene expression analysis data, J774A.1 cells express several types of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) receptors and  $\beta_2$ -adrenergic receptors, which activate  $\text{G}\alpha\text{s}$  (data not shown). To examine the effect of loss of  $\text{G}\beta\gamma$  on  $\text{G}\alpha\text{s}$ -mediated signaling, we measured cAMP production stimulated with PGE<sub>2</sub> and isoproterenol. Both agonists induced cAMP generation in wild-type cells and single shRNA-containing cells, but not in the cells containing both shRNAs of Gβ1 and Gβ2 (Fig. 2C). On the other hand, forskolin, which directly activates adenylyl cyclases, induced cAMP production in all cell types. Taken together, the data suggest that  $\text{G}\beta$ -subunits are also essential for  $\text{G}\alpha\text{s}$ -mediated signaling.

**Migration Activity Is Impaired in Cells Lacking  $\text{G}\beta$  Proteins.** Migration of immune cells to most chemokines depends on members of the pertussis toxin-sensitive  $\text{G}\alpha\text{i}$  signal transducing gene family (28). The activated receptor releases the  $\text{G}\beta\gamma$ -subunits from the  $\text{G}\alpha\text{i}$  heterotrimer, and they in turn activate a variety of proteins that are directly involved in initiating chemotaxis (29). We used Boyden chamber assays to examine migration activity of the cells to C5a, whose receptor is expressed in J774A.1. As shown in our previous report (15), control and Gβ1 shRNA-containing cells migrated to C5a in a dose-dependent manner. However, manifold fewer migrating Gβ2 shRNA-expressing cells were observed as a result of C5a stimulation. For the cells containing both shRNAs that simultaneously eliminated Gβ1 and Gβ2, we could not detect any chemotaxis toward C5a (Fig. 3A). This result further supports the conclusion that all signaling pathways involved in migration to C5a were impaired in the absence of  $\text{G}\beta$  proteins.

Phosphatidylinositol 3-kinase  $\gamma$  (PI3K $\gamma$ ) plays a major role in cell





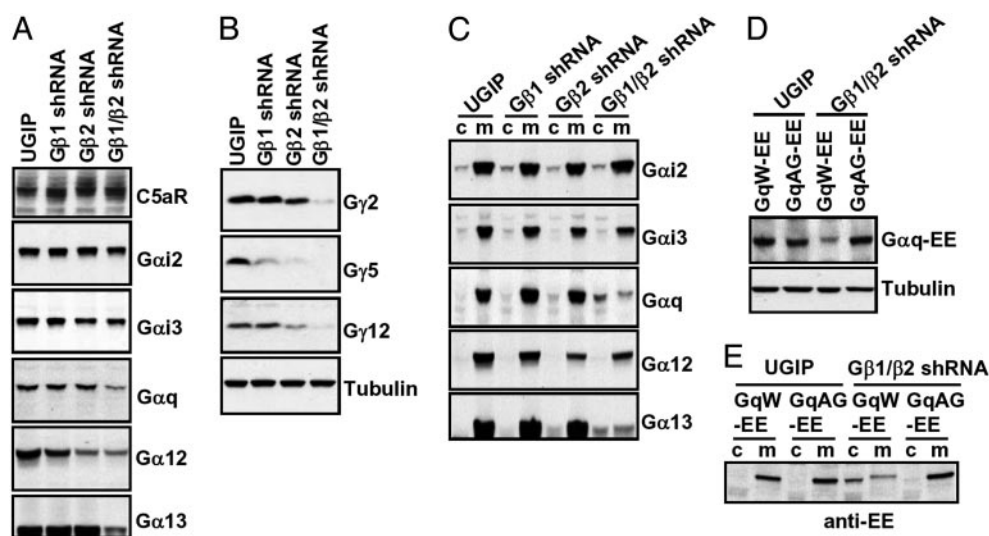
**Fig. 4.** Effects of G $\beta$  silencing on actin polymerization by C5a. Cells were treated with C5a for 2 min, fixed with 4% paraformaldehyde, and stained with Texas red-conjugated phalloidin. C5a induced actin polymerization in control and the cells containing either G $\beta$ 1 or G $\beta$ 2 shRNA. However, actin polymerization was not detected in the cells containing both G $\beta$ 1 and G $\beta$ 2 shRNA.

migration by interacting with G $\beta\gamma$ -subunits (30, 31). PI3K produces phosphatidyl inositol-3,4,5-tris-phosphate, which induces membrane translocation and phosphorylation of Akt. We examined Akt phosphorylation, an event reflective of PI3K activation. The Akt phosphorylation on Ser-473 by C5a was observed after 2 min of stimulation and was sustained for >30 min in C5a-treated control cells and G $\beta$ 1 shRNA-containing cells. In G $\beta$ 2 shRNA-containing cells, phosphorylation occurred after 2 min but disappeared within 15 min, implying that G $\beta$ 2 is important for extended Akt phosphorylation. However, Akt phosphorylation was not detected at all in the cells containing both shRNAs (Fig. 3B).

**C5a Cannot Increase Actin Polymerization in Cells Depleted of G $\beta$ -Subunits.** Polymerization of actin filaments is an indispensable intracellular event for cell migration in cells responding to chemokines. Rho GTPases are implicated in this process (32). Rho is implicated in the actin polymerization and formation of focal adhesion complexes, resulting in actomyosin assembly and the contractile tension necessary for movement (33). G $\alpha$ 12/13 medi-

ates the Rho activation signal from chemokine receptors by stimulating guanine nucleotide exchange factor, p115Rho-GEF (34). To determine the effect of G $\beta$  depletion on G $\alpha$ 12/13-mediated signaling by chemokine receptors, we stimulated the cells with C5a. Actin polymerization occurred in control cells and single shRNA containing cells stimulated with uniform C5a for 2 min (Fig. 4). However, polymerized actin filaments were not observed in cells containing shRNA directed against both G $\beta$ 1 and G $\beta$ 2. We observed cells for 30 min in 5-min intervals to exclude delayed responses to stimulation, but no actin polymerization was detected (data not shown).

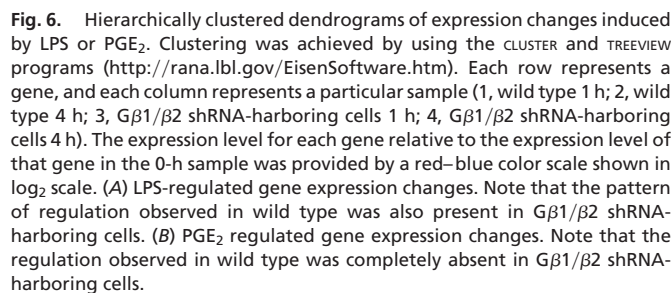
**G $\beta$ -Subunits Affected the Expression of G $\alpha$  and G $\gamma$ .** It is well known that the individual members of the G protein heterotrimers are necessary for the stability of their binding partners (10, 15). According to Western blotting and FACS analysis with anti-C5a receptor antibody, expression levels of the receptor were not changed in control and shRNA-containing cells, and membrane localization was maintained (Fig. 5A), indicating that the absence of



**Fig. 5.** Expression of C5a receptors, G $\alpha$ , and G $\gamma$  in the absence of G $\beta$ -subunits. (A and B) Cells were lysed with lysis buffer containing 0.1% SDS, and expression of each protein was analyzed with specific antibodies. Protein expression was normalized to tubulin levels in total cell extracts. (C) The distribution of G $\alpha$ -subunits between cytosol (c) and membrane (m) was analyzed by Western blotting. (D and E) The effect of myristylation on expression and localization of G $\alpha$ q. Adenovirus containing EE-tagged forms of wild-type G $\alpha$ q and G $\alpha$ qAG (myristylation form) were transduced into J774A.1 cells. The cells were harvested, and the expression of EE-tagged G $\alpha$ q was detected with anti-EE.

Interaction with G $\beta\gamma$ -subunits and G $\alpha$  lipid modification myristylation and palmitoylation are required for the membrane localization of G $\alpha$ -subunits (21, 35–37), but there is some controversy about the relative role of G $\beta\gamma$  in the localization of G $\alpha$ -subunits. For example, release of G $\beta\gamma$  from the membrane did not prevent retention of Gao in the growth cone of nerve cells (38). Several G $\alpha$ -subunits can be localized in the Golgi membrane in the absence of G $\beta\gamma$ -subunits (39). It has been suggested that double lipid modification is sufficient to maintain G $\alpha$  at the plasma membrane; however, in the absence of interaction with G $\beta\gamma$ -subunits, the G $\alpha$  subunit protein may not be effective. In this study, cells were separated into cytosol and membrane to identify subcellular localization of G $\alpha$ -subunits. As shown in Fig. 5C, the majority of G $\alpha$ -subunits in mock-treated cells were detected in membrane fractions. In the double knockdown cell, the membrane localization of G $\alpha$ i2, G $\alpha$ i3, and a portion of the G $\alpha$ 12 protein was not changed. On the other hand, a large fraction of the G $\alpha$ q and G $\alpha$ 13 protein was absent and residual amounts were detected in the cytosol fractions of the G $\beta$ -depleted cells. Interestingly, G $\alpha$ i family members have dual lipidation (myristylation and palmitoylation), but G $\alpha$ q and G $\alpha$ 13 lack a myristylation site and have only a palmitoylation residue. To determine the effect of lipid modification on the expression of G $\alpha$ -subunits in the G $\beta\gamma$ -deficient cells, we followed the work of Evanko *et al.* (21) and constructed recombinant G $\alpha$ q (G $\alpha$ qAG) containing dual lipidation sites by substituting Ala with Gly in N-terminal region and using an adenoviral vector to mediate gene expression. After adenovirus infection into control cells and G $\beta$ -deficient cells, subcellular fractionation was performed. The expression of G $\alpha$ qAG was not changed in both cells, although wild-type G $\alpha$ q was down-regulated in cells lacking G $\beta$ -subunits (Fig. 5D). Furthermore, G $\alpha$ qAG was detected in the membrane fraction but not in cytosol fraction, which is similar to its expression pattern in control cells (Fig. 5E). The results further support the notion that dual lipid modification, myristylation in addition to palmitoylation, even in the absence of G $\beta\gamma$ , is sufficient for membrane localization of the G $\alpha$ q-subunits and perhaps for the G $\alpha$ i-subunits as well. Once bound to the membrane, the G $\alpha$ -subunit proteins can remain in a stable form. The G $\beta\gamma$ -subunits also play a critical role in stabilizing and directing the singly modified G $\alpha$ -subunits to a suitable membrane region for interaction with the GPCR (36, 37). The exception not covered by this rule is G $\alpha$ 12. Although its level is markedly decreased, the residual protein does remain associated with the membrane fraction. However, our fractionation procedure does not allow us to distinguish between plasma membrane, Golgi, or endoplasmic reticulum. G $\alpha$ 12 could interact with other components and be stabilized in the Golgi fraction.

**Gβ-Subunits Were Not Involved in Phagocytotic Activity or Growth of J774A.1 Cells.** In studies of *Dictyostelium discoidium*, deletion of Gβ resulted in impaired phagocytosis and chemotaxis (14). Using opsonized zymosan particles with Ig, we examined phagocytotic activity of the cells. Contrary to the results in *Dictyostelium*, the activity was similar in all cells (data not shown).



Although phagocytosis depends on actin polymerization and rearrangement, immune cells recognize opsonins such as Ig and complement that bind to foreign bodies through cognate receptors. These receptors presumably triggered signaling pathways for phagocytosis without activating heterotrimeric G proteins.

**Gene Expression Induced by PGE<sub>2</sub> and UTP Was Blocked in the Absence of Gβ-Subunits.** Extracellular stimulants may induce various cellular responses by regulating specific gene expression. To observe the effect of the absence of Gβ on ligand-dependent gene regulation, we examined the gene expression in J774A.1 cells stimulated with LPS (100 ng/ml LPS and 100 pM LPS-binding peptide), 10 μM

PGE<sub>2</sub>, and 10  $\mu$ M UTP by using microarrays that monitored the expression of  $\approx 11,000$  transcripts. By the criterion of a  $\geq 2$ -fold change in expression in all experiments, exposure of the cells to LPS for 1 h resulted in the up-regulation of  $>100$  transcripts in control cells. LPS treatment of cells lacking G $\beta$ s for 1 h led to a similar pattern of gene expression as in the control cells. The expression of  $>200$  genes was changed in response to LPS exposure for 4 h in cells lacking G $\beta$ s. Although the overall pattern of gene expression was similar to that of the control cells, there were subtle differences in the changes in transcript levels induced by LPS treatment in the cells lacking G $\beta$  protein (Fig. 6A and data not shown). On the other hand, PGE<sub>2</sub> regulated the expression of 60 genes after 1 h of treatment and 180 genes after 4 h (Fig. 6B). However, there was no change in transcript level in the cells depleted of G $\beta$ -subunits. Thus, whereas G $\beta$ -subunits are not necessary to mount the Toll receptor response pattern, the presence of G $\beta$  protein is absolutely required for the PGE<sub>2</sub> response. There is evidence to suggest that some of the subsequent steps in LPS-stimulated gene expression can be modulated by GPCR signaling. Thus, for example, PGE<sub>2</sub> receptors are up-regulated by LPS treatment and, when activated, attenuate LPS-induced gene expression in macrophage cells (26, 40). Extended treatment with LPS may recruit G protein-signaling pathways and modify later gene expression. A more complete analysis of the effects of the loss of G $\beta$ -subunit protein and the subsequent interruption of G protein-mediated signaling on ligand induced responses is required.

## Discussion

J774A.1 cells primarily express the G $\beta$ 1 and G $\beta$ 2 isoforms. We have previously shown that “knockdown” of gene expression of G $\beta$ 2 can lead to migration defects to some chemokines (15). The loss of both G $\beta$ 1- and G $\beta$ 2-subunits leaves the cells with essentially no G $\beta$ . Because G $\gamma$  is stable only in a complex with G $\beta$ , the loss of G $\beta$  resulted in the collateral loss of the G $\gamma$ -subunits. Interestingly the G $\alpha$ -subunit proteins that did not contain multiple lipid modification residues and thus did not reach the membrane were also destabilized. It is not clear whether the membrane-associated

G $\alpha$ -subunits have any alternative activity, but it would be surprising if they could be activated by GPCRs. On the other hand, there might be other exchange factors that could activate the residual G $\alpha$ -subunits. At present, there is no evidence to support this conjecture. In fact, all of the data suggest that, although GPCRs remain intact, the absence of  $\beta\gamma$ -subunits results in the specific inactivation of all of the tested G protein gene family-mediated signaling pathways. The G $\alpha$ s pathway includes gene activation and cAMP generation by PGE<sub>2</sub> and isoproterenol stimulation. The G $\alpha$ i pathway includes chemotaxis to C5a. The G $\alpha$ q pathway is responsible for Ca<sup>2+</sup> release in response to UTP, and the G $\alpha$ 13 pathway is critical for actin polymerization.

We do not have a comprehensive picture of how the cells adapt to the absence of the G protein-mediated pathways. However, it is clear that, when not stressed, they behave relatively normally. They can grow at rates similar to those of parent cells, can generate complex responses to ligands such as LPS, and can organize complex cellular movements and shape changes required for phagocytosis. On the other hand, the transcriptional regulation as well as the elevation of cAMP levels mediated by the application of isoproterenol or PGE<sub>2</sub>, which utilizes the Gs pathway, were completely eliminated.

The data with the G $\beta$ 1/G $\beta$ 2 double knockdowns demonstrate how the individual components of a protein complex can depend on protein folding and assembly for both their stability and function. In addition, the data show that, in the absence of a functioning G protein-mediated signaling system, cells can grow and other signaling pathways can function. By using a variety of ligand addition protocols (as described by the Alliance for Cell Signaling), it may be possible to use the G $\beta$ 1/G $\beta$ 2-depleted cells in transcript analysis experiments to determine the nature of signal pathway interaction.

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